DETAILED METHODS

Mouse models of AKI

Male C57BL/6 mice weighing 22-24g were obtained from the Southern Medical University Animal Center (Guangzhou, China) and housed in standard environment with regular light/dark cycles and free access to water and chow diet. Mouse models of AKI were established by IRI or cisplatin, respectively. For ischemic AKI, bilateral renal pedicles were clipped for 30 minutes in mice using microaneurysm clamps, using an established protocol.[1] Reperfusion of the kidneys was visually confirmed after removal of the clamps. The incision was then closed and animals were allowed to recover. During the ischemic period, body temperature was maintained between 37~37.5°C using a temperature-controlled heating system. Mice were sacrificed at 30 hours post-IRI and blood and tissue samples were collected. Pilot experiments showed that at 30 hours after bilateral IRI, renal injury and dysfunction reached the peak, and animals started to die in this model.

For toxic AKI, mice were subjected to a single intraperitoneal injection of cisplatin (Sigma, St. Louis, MO). Cisplatin was freshly prepared at 1 mg/ml in saline and administered by intraperitoneal injection at a dose of 40 mg/kg as described elsewhere.[2] For animal survival experiments, 25 mice were used in each group and survival of animals monitored for 72 hours. In another set of experiment, mice were sacrificed at 36 hours after cisplatin injection, and serum and kidney samples were collected for various analyses. All animal experiments were approved by the Animal Ethic Committee at the Nanfang Hospital, Southern Medical University.

Human Samples

All studies involving human samples were approved by the Ethic Committee on Human Subjects of the Nanfang hospital, Sothern Medical University. The study participants provided written informed consent. Healthy subjects (26 cases) without preexisting clinical conditions were recruited and blood collected. Patients receiving elective cardiac surgery who developed AKI were also recruited. Among them, 30 patients developed severe AKI, defined as doubling of serum creatinine or a need for dialysis (consistent

with KDIGO stage 2 or 3). Another cohort of 30 patients developed mild AKI, defined as an increase in serum creatinine by 26.5 mmol/L (0.3 mg/dl) within 48 hours or a 50% increase in serum creatinine from the preoperative level within 7 days (KDIGO criteria). Plasma samples were obtained at 48 hours after surgery from the above patients at the Nanfang Hospital, Southern Medical University. Exclusion criteria included exposure to nephrotoxin (i.e., contrast media, aminoglycoside antibiotics, vancomycin, and nonsteroidal anti-inflammatory drugs except aspirin) within 4 weeks before surgery, preexisting advanced CKD (chronic dialysis, renal transplantation, or preoperative eGFR＜30 ml/min per 1.73 m2), and urinary tract infection or obstruction. The demographic and clinical data of the healthy subjects and patients were presented in Supplementary Table 1. Human kidney biopsy sections from AKI patients (2 cases) were also obtained from diagnostic renal biopsies performed at the Nanfang Hospital and used for immunohistochemical staining for TNC.

TNC ELISA

Human Tenascin-C Large (FNIII-B) Assay Kit was purchased from the Immuno-Biological Laboratories (IBL) (Code No.27767; IBL Company, Gunma, Japan). This assay is a solid phase sandwich ELISA using two kinds of highly specific antibodies for human TNC and it can detect TNC high molecular weight variants including FNIII-B domain. Plasma TNC was measured according to the assay procedures specified by the manufacturer, and expressed as nanograms per milliliter.

Knockdown of TNC in vivo

Knockdown of TNC expression in vivo was carried out by shRNA-mediated approach. Male C57/BL6 mice were divided into three groups (n = 6 in each group): (*1*) sham-operated mice, (*2*) IRI mice receiving control shRNA, and (*3*) IRI mice receiving TNC-shRNA. Two days prior to IRI, mice were injected with either pLVX-shTNC or control plasmids via tail vein using hydrodynamic-based gene delivery technique, as described previously.[3] Mice were then subjected to IRI as described above. Animals were sacrificed at 30 h

after IRI, and serum and kidney tissues collected for analyses. Similar approach was used to knockdown TNC expression in cisplatin model in vivo. Briefly, two days prior to cisplatin injection, mice were injected with either pLVX-shTNC or control plasmids via tail vein. Mice were sacrificed at 36 h after cisplatin injection.

Determination of serum creatinine and BUN

Serum was collected from mice at 30 hours after IRI. Serum creatinine level and blood urea nitrogen (BUN) were determined by an automatic chemistry analyzer (AU480; Beckman coulter, Pasadena, California). The levels of serum creatinine and BUN were expressed as mg/dl.

Cell culture and treatment

Human proximal tubular epithelial cells (HKC-8) were provided by Dr. L. Racusen (Johns Hopkins University, Baltimore, MD). Cell culture was carried out according to the procedures described previously.[4] HKC-8 cells were treated with cisplatin (Sigma, St. Louis, MO) or staurosporine (Sigma) at the specified concentrations to induce cell apoptosis. For some experiments, HKC-8 cells were pretreated with human recombinant TNC (R & D Systems, Minneapolis, MN) at 50 ng/ml for 1 hour, followed by incubation with cisplatin or staurosporine. Whole-cell lysates were prepared and subjected to Western blot analyses. Some cells were also harvested and analyzed with PE-conjugated Annexin V (AV)-labeled apoptotic cells detection kit (AP104-100-kit; Multi Sciences biotech, Hangzhou, China), followed by analyzing with flow cytometry (FACSCanto II; Becton Dickinson, San Jose, CA).

Hypoxia/reoxygenation injury

In vitro ischemia model of kidney tubular cells was established by hypoxia/reoxygenation protocol. Briefly, cells were cultured to 70–80% confluence in complete medium and changed to serum-free medium for 24 hours, and then incubated in glucose-free medium in a tri-gas incubator (94% N2, 5% CO2 and 1.0% O2) at

37 °C for 24 hours. Subsequently, cells were incubated in complete medium under normal (5% CO2 and 95% air) conditions for 2 hours for reoxygenation, and then harvested for various analyses.

TUNEL assays

TUNEL staining for apoptotic cells was performed on the paraffin-embedded mouse kidney sections (4 μm thickness) using a standard DAB incorporation kit (KGA7042; KeyGen, Nanjing, China) according to the instructions specified by the manufacturer. HKC-8 cells were cultured on the coverslips and subjected to various treatments as indicated. The slides were then incubated in TDT buffer containing the TdT enzyme and HRP-conjugated dUTP. Apoptotic cells were stained in brown by HRP-catalyzed DAB reaction. HKC-8 cells were fixed with cold methanol:acetone (1:1) for 15 min, then incubated in TDT buffer containing the TdT enzyme and FITC-conjugated dUTP according to the manufacturer's protocol (QIA39; Merck Millipore, Billerica, MA). Apoptotic cells were detected after counterstaining with Hoechst 33258 (Merck Millipore). The images were collected under fluorescent microscopy (Leica TCS SP2 AOBS, Cambridge, UK).

Western blot analysis

Protein expression was analyzed by Western blot analysis as described previously.[5] The primary antibodies used were as follows: anti-TNC (ab108930; Abcam, Cambridge, MA), anti-Bax (sc-493;Santa Cruz Biotechnology, Santa Cruz, CA), anti-Kim-1 (BA3537; Boster Biological Technology, Wuhan, China), anti-β-catenin (#610154; BD Transduction Laboratories, San Jose, CA), anti-FasL (sc-6237; Santa Cruz Biotechnology), anti-caspase 3 (9662s; Cell Signaling Technology, Danvers, MA), anti-P53 (sc-126; Santa Cruz Biotechnology), anti-Parp-1 (#9542S; Cell Signaling Technology), rabbit polyclonal anti-active β-catenin (4270s; Cell Signaling Technology), anti-β-catenin (610154; BD), anti-Wnt1 (ab15251; Abcam), anti-Wnt4: (AF475; R&D), mouse anti-TBP (ab818; Abcam), anti-survivin (PB0377; Boster Biological Technology, Wuhan, China), anti-Wnt3a (SAB2108434; Sigma-Aldrich), anti-GFP (ab13970; Abcam), anti-HA: (ab9110; Abcam) and mouse monoclonal anti-α-tubulin (MAB1501; Millipore, Billerica, MA).

Reverse transcriptase (RT)-PCR

Total RNA was prepared using TRIzol RNA isolation system (Life Technologies, Grand Island, NY) according to the manufacturer's instruction. The first strand of complementary DNA was synthesized using 1 μg of RNA in 20 μl of reaction buffer using AMV-RT and random primers at 42℃ for 60 min. PCR amplification was performed using a HotStar Taq Master Mix kit (Qiagen, Valencia, CA). The sequences of primers of mouse TNC were as follows: 5'-ATGCCACTCCAGACGGTTTC -3' and 5'-GCTGACTGTGGCTGCATTTT-3'. The sequences of the primers for β-actin were described previously.[3]

Histology and immunohistochemical staining

Paraffin-embedded mouse kidney sections (4 μm thickness) were prepared by a routine procedure. Immunohistochemical staining was performed using routine protocol. Antibodies used were as follows: rabbit polyclonal anti-β-catenin (ab15180; Abcam), rabbit monoclonal anti-TNC (ab108930; Abcam), rabbit polyclonal anti-caspase 3 (9662s; Cell Signaling Technology). Some human kidney biopsy sections were obtained from diagnostic renal biopsies performed at the Nanfang Hospital and stained with rabbit monoclonal anti-TNC (Abcam). All studies involving human kidney sections were approved by the Institutional Ethics Committee at the Nanfang Hospital, Southern Medical University.

Immunoprecipitation

The interaction of TNC and Wnts was determined by co-immunoprecipitation, as previously described.[4] HKC-8 cells were transfected with different HA-tagged Wnt expression plasmids (pHA-Wnt1 or pHA-Wnt4) and incubated in the absence or presence of TNC for 24 hours. Cell lysates were immunoprecipitated overnight at 4°C with anti-TNC antibody (R&D System) and protein A/G plus agarose (sc-2003; Santa Cruz Biotechnology). The precipitated complexes were washed three times with lysis buffer and boiled for 5 minutes in SDS sample buffer, followed by immunoblotting with rabbit polyclonal anti-Wnt1 (ab15251;

Abcam), rabbit polyclonal anti-Wnt4 (sc-13962), respectively. In the reciprocal experiments, cell lysates were immunoprecipitated with anti-HA antibody, followed by immunoblotting with anti-TNC antibody.

Luciferase Assay

The effect of TNC on Wnt-mediated gene transcription was assessed by using the TOP-Flash reporter plasmid containing two sets of three copies of the T cell factor (TCF)-binding site at the upstream of the thymidine kinase minimal promoter and luciferase cDNA (Millipore, Billerica, MA). HKC-8 cells were transfected using Lipofectamine 2000 reagent with TOP-Flash plasmid and Wnt1 expression vector (pHA-Wnt1) in the absence or presence of TNC. An internal control reporter plasmid (0.1 mg) Renilla reniformis luciferase driven under thymidine kinase promoter (Promega, Madison, WI) was also co-transfected for normalizing the transfection efficiency. Luciferase assay was performed using a dual luciferase assay system kit according to the manufacturer's protocols (Promega). Relative luciferase activity (arbitrary units) was reported as fold induction over the controls after normalizing for transfection efficiency.

Nuclear and Cytoplasmic Fractionation

For preparation of nuclear and cytoplasmic protein after subcellular fractionation, HKC-8 cells were collected and washed twice with cold PBS. Nuclear and cytoplasmic protein was prepared using a commercial kit (BB-3102-50T; BestBio, Shanghai, China) according to the protocols specified by the manufacturer.

Preparation of kidney tissue scaffold

Kidney tissue scaffolds (KTS) were prepared from sham controls or ischemic kidneys at 30 hours after IRI, according to protocols reported previously.[5] Briefly, at 30 hours after IRI, groups of mice were euthanized, and kidney was perfused *in situ* using a saline solution to remove the blood. Each kidney was cut into 3 to 4 slices of uniform thickness along the sagittal plane. The kidney slices were then immersed in 100 ml of ultrapure water, followed by shaking for 30 minutes for three times. The slices were then incubated with 100

ml of 0.02% trypsin/0.05% EDTA solution for 1 hour at 37°C, and rinsed with water and transferred to a flask with 100 ml of 3% Triton X-100 solution, followed by shaking for 12 hours. After brief washes, the kidney slices were incubated with 100 ml of 4% deoxycholic acid solution on a shaker for 5 hours. The kidney scaffolds were thoroughly rinsed with water to remove residual surfactant, and stored in ultrapure water in the refrigerator until further usage.

Statistical analyses

All data examined were expressed as mean ± SEM. Statistical analysis of the data was carried out using SPSS 13.0 (SPSS Inc, Chicago, IL). Comparison between groups was made using one-way ANOVA followed by Student-Newman-Kuels test or Dunnett's T3 procedure. The animal survival curve was analyzed by Log-rank test. $P < 0.05$ was considered significant.

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